Exhibit 1

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Hypoallergenic Variants of the *Parietaria judaica* Major Allergen Par j 1: A Member of the Non-Specific Lipid Transfer Protein Plant Family

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Key Words

Parietaria judaica · Par j 1 recombinant allergen · Hypoallergenic recombinant Par j 1 mutants · Skin prick test · Immunoglobulin E · Peripheral blood mononuclear cell proliferation ·

Abstract

Background: Par j 1 represents a major allergenic component of *Parietaria judaica* (Pj) pollen, since it is able to induce an immunoglobulin E (IgE) response in 95% of Pj-allergic patients. It belongs to the non-specific lipid transfer protein family, sharing with them a common three-dimensional structure. ***Methods:*** Disulphide bond variants of the recombinant Par j 1 (rPar j 1) allergen were generated by site-directed mutagenesis, and the immunological activity of rPar j 1 and its conformational mutants was compared with the use of the skin prick test (SPT). The ability to bind IgE antibodies was evaluated by Western blot, ELISA and ELISA inhibition. T cell reactivity was measured by peripheral blood mononuclear cell proliferation assay. ***Results:*** The disruption of Cys14–Cys29 and Cys30–Cys75 bridging (PjA mutant) caused the loss of the majority of specific IgE-binding activity. Additional disruption of the Cys4–Cys52 bridge (PjC mutant) and the latter Cys50–Cys91 bridge (PjD mutant) led to the abolition of IgE-binding activity. On the SPT, PjB (lacking the Cys4–Cys52 and Cys50–Cys91 bridges) was still capable of triggering a type I hypersensitive reaction in 9 out of 10 patients, and PjA in 3 out of 10 patients, while PjC and PjD did not show any SPT reactivity. All the mutants preserved their T cell reactivity. ***Conclusion:*** Recombinant hypoallergenic variants of the rPar j 1 allergen described herein may represent a useful tool for improved immunotherapy.

Introduction

Atopy is a genetic immune disorder affecting an increasing number of people living in industrialised countries. It is characterised by a group of diseases with different severity, like seasonal allergy, atopic dermatitis and more serious conditions such as anaphylaxis. Immunoglobulin E (IgE) antibodies represent one of the main actors in this reaction, since the cross-linking of mast cell-bound IgEs by allergen leads to the release of inflammatory mediators responsible for the immediate allergic reaction [1]. In addition, IgE antibodies have been shown to be involved in the uptake and presentation of allergens by antigen-presenting cells through their high- and low-affin-

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ity receptors [2–4]. The capture and processing of allergens by surface IgE seems to be a crucial event even in the determination of the profile of cytokine production by T helper (Th) cells, since a report suggests a different route for non-IgE-binding variants of allergens [5]. In this scenario, the design of genetically modified hypoallergenic variants of allergens with low or absent IgE affinity has been evaluated as a new strategy in the development of a more efficient and safer allergen specific immunotherapy (SIT).

SIT has been shown to be the only treatment effective in many clinical trials [6]. Successful immunotherapy is associated with an increase in IL-10 production and a decrease in the IgE/IgG4 ratio in bee venom, grass pollen and house dust mite immunotherapy [7–9]. However, commercial crude extracts are usually a partially purified mixture of proteins whose allergenic components are difficult to standardise. In addition, their administration to patients can lead to sensitisation towards new allergens and, rarely, to dangerous anaphylactic reactions. The use of recombinant DNA technologies has allowed the isolation and expression of many allergens, and their immunological characterisation suggests that they might be used as substitutes for native counterparts [see ref. 10 for a review].

Pollen allergens are usually low-molecular-weight proteins capable of inducing IgE production by B cells. In particular, *Parietaria judaica* (Pj) pollen represents one of the main sources of allergens in the Mediterranean area [11], and Pj IgE-specific antibodies have also been detected in certain areas of southern UK, America and Australia [12]. The composition of allergenic extracts of Pj pollen has been extensively studied, and the two major allergens and isoforms have been cloned and immunologically characterised [13–19]. The Par j 1.0102 molecule is a 176-amino acid protein with a 37-amino acid NH_2 terminal signal peptide giving a 139-amino acid mature processed protein with a deduced molecular mass of 14,400 D. It is a major allergen, since it induces a response in 95% of Pj-allergic patients, and the recombinant protein produced in *Escherichia coli* shows a pattern of reaction similar to the native counterpart [17, 18, 20]. Sequence analysis showed a high level of homology with a family of plant proteins named non-specific lipid transfer proteins (nsLTPs) for their ability to transport lipids through the membrane in vitro [21]. A three-dimensional model of Par j 1 has been proposed by structural homology with the soybean nsLTP, showing an α-α-α-α-β structure with four disulphide bridges necessary for the correct folding of the protein, according to the invariant order Cys4–Cys52, Cys14–Cys29, Cys30–Cys75 and Cys50–Cys91 [22]. The same study reported the mapping of a conformational B cell epitope and the importance of the cysteine pairing in the formation of an immunodominant IgE epitope [22].

Starting from these observations, we decided to test the contribution of the four disulphide bonds to the allergenicity of the Par j 1 protein by designing genetically modified molecules with reduced or absent ability to bind IgE in order to develop new strategies for improved SIT.

Materials and Methods

Site-Directed Mutagenesis

Par j 1 clone, expressing the wild-type Par j 1.0102 allergen, was generated by polymerase chain reaction (PCR) amplification of 1 of the P5 clone (EMBL accession number X77414) at the follow$_{10\times}$ conditions: 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min for 30 cycles using the primers P5 forward (mapping from nucleotide 1 to nucleotide 21; 5' ATT gga tcc CAA GAA ACC TGC GGG ACT ATG 3') and P5 reverse (mapping from nucleotide 405 to nucleotide 423; 5' ATT aag ctt GGC TTT TTC CGG TGC GGG 3') (lower-case letters indicate the restriction enzyme sites used for cloning). The PCR fragment was purified, digested with *Bam*H I and *Hind* III restriction enzymes and cloned in pQE30 vector (Qiagen) previously digested with the same enzymes. The PjA mutant (Cys29→Ser and Cys30→Ser) was generated using the Transformer Site-Directed Mutagenesis kit (Clontech) following the manufacturer's instructions and using the oligonucleotide (mapping from nucleotide 88 to nucleotide 105) 5' GGG AGC AGC AGC GGC GCC 3' (bold letters indicate the mutated nucleotides) and the Par j 1 sequence as a template. The PjB mutant (Cys50→Ser and Cys52→Ser) was generated by PCR using the primers P5(50–52) (mapping from nucleotide 91 to nucleotide 165: 5'CGT Gct gga gCG GCG CCA AAA GAT TGG ACG GGG AGA CGA AGA CGG GGC CGC AGA GGG TGC ACG CTA GTG AGA GCA TC 3') and P5 reverse oligonucleotide and 1 ng of the Par j 1 clone as a template (lower-case letters indicate the restriction enzymes used for cloning, bold letters indicate the mutated nucleotides). The PCR fragment was digested with *Par I* *Hind* III restriction enzymes and ligated with the *Par I*-*Hind* III linearised plasmid vector containing the Par j 1 sequence (expressing the first 31 amino acids of the wild-type Par j 1.0102 allergen). The PjC mutant (Cys4→Ser, Cys29→Ser and Cys30→Ser) was generated by PCR amplification using the PjA variant as a template. The cysteine residue at position 4 was mutated by PCR using the oligonucleotides P5(triple) (mapping from nucleotide 1 to nucleotide 17; 5' GCA gga tcc CAA GAA ACC AGC GGG AC 3') and P5 reverse oligonucleotide (lower case letters indicate the restriction enzyme sites used for cloning and bold letters indicate the mutated nucleotide). After purification, the PCR fragment was digested with *Bam*H I and *Hind* III enzymes and cloned in pQE30 vector previously digested with the same restriction enzymes. The PjD mutant (Cys29→Ser, Cys30→Ser, Cys50→Ser and Cys52→Ser) was generated using the Transformer Site-Directed Mutagenesis kit (Clontech) following the manufacturer's instructions and using the synthetic oligonucleotide (mapping from nucleotide 88 to nucleotide 105)

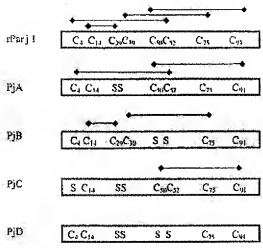

Fig. 1. Schematic representation of the wild-type rPar j 1 allergen and its four disulphide bond variants, i.e. PjA (lacking Cys14–Cys29 and Cys30–Cys75 bonds), PjB (lacking Cys50–Cys91 and Cys4–Cys52 bonds), PjC (lacking Cys4–Cys52, Cys14–Cys29 and Cys30–Cys75 bonds) and PjD (lacking Cys4–Cys52, Cys14–Cys29, Cys50–Cys91 and Cys30–Cys75 bonds). C indicates cysteine residues and the subscript numbers indicate their position on the wild-type sequence; S indicates Ser→Cys substitutions. The horizontal bars above the sequences indicate the presence of disulphurous bridges according to the following order: Cys4–Cys52, Cys14–Cys29, Cys30–Cys75, Cys50–Cys91.

5′ GGG AGC AGC AGC GGC GCC 3′ (bold letters indicate the mutated nucleotides) and the PjB variant as a template. All clones were sequenced following the method of Sanger et al. [23], and the mutations and the open reading frames were confirmed (fig. 1).

Induction and Purification of Recombinant Proteins

The recombinant clones (NM15 strain, Qiagen) were grown overnight in 2YT broth (Bacto-tryptone 16 g/l, Bacto-yeast 10 g/l, NaCl 5 g/l, pH 7.0). A 1/40 dilution was grown for 1 h at 37°C and, after that, induced with 1 m*M* isopropylthio-β-galactoside for 4 h at 37°C. Cells were harvested by centrifugation and the recombinant proteins were purified using the HisTrap kit (Pharmacia) following the manufacturer's instructions. Recombinant proteins, binding the HiTrap chelating column, were eluted using a buffer containing 20 m*M* phosphate buffer, pH 7.4, 0.5 *M* NaCl, 8 *M* urea and 500 m*M* imidazole; fractions were analysed by 16% SDS-PAGE and Coomassie brilliant blue staining. Fractions containing the purified protein were then diluted 1/100 in a buffer containing 20 m*M* phosphate buffer, pH 7.4, 0.5 *M* NaCl and 20 m*M* imidazole to allow refolding of the protein, reloaded on the HiTrap column and eluted with a buffer with no denaturing agents (20 m*M* phosphate buffer, pH 7.4, 0.5 *M* NaCl and 500 m*M* imidazole). Recombinant proteins were then desalted using a centrifugal filter device (Centriprep, Millipore) and analysed for their ability to bind human IgE from Pj-allergic patients by Western blot, as previously described [22]. After that, membranes were stripped and reprobed with a His tag-specific reagent (INDIA™ His-probe-HRP, Pierce, USA) to check that the IgE-allergen complex was specific for the recombinant fused proteins. The concentration of the recombinant proteins was determined by densitometric analysis of SDS-PAGE gels stained with Coomassie brilliant blue (fig. 2C).

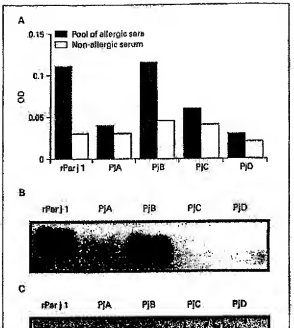

Fig. 2. ELISA detection (**A**) and Western blot analysis (**B**) showing the IgE-binding activity of rPar j 1 and its disulphide bond variants using a pool of sera (n = 30) from monosensitive Pj-allergic patients. A non-allergic subject was tested as a negative control for the ELISA. **C** Coomassie brilliant blue staining of the recombinant proteins used.

ELISA Detection

ELISA detection was performed by adding 200 µl of a solution containing 5 µg/ml of antigen in coating buffer (sodium carbonate buffer, pH 9.5) to each well of polystyrene plates overnight at room temperature. After several washing steps (1 × PBS, 0.1% Tween 20), the plates were saturated with a solution containing 5% BSA and 0.5% Tween 20 in coating buffer. After washing, 200 µl of serum (1/5 dilution) from Pj-allergic patients or from a non-allergic subject were incubated for 4 h at room temperature. Bound IgE antibodies were

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detected with a goat anti-human IgE-HRP conjugate (Biosource International) diluted at a concentration of 0.5 ng/ml in 1 × PBS, 0.25% BSA and 0.1% Tween 20 for 1 h at room temperature. After several washes, colorimetric reaction was developed by adding 0.2 ml/well of substrate solution (0.4 mg/ml o-phenylenediamine in 0.1 *M* citrate buffer). Optical density was read at 495 nm in a BIO-RAD microplate reader.

Rabbits were immunised by PRIMM srl (Milan, Italy) using the recombinant Par j 1 (rPar j 1) allergen. As a control, rabbit polyclonal antibodies were analysed on a Western blot using a Pj crude extract detecting a band of about 14,000 D, corresponding to the Par j 1 native molecular weight (data not shown). ELISA plates were coated at the same conditions as described above. Rabbit pre-immune and immune sera were diluted at a concentration of 6 ng/ml, and 200 µl of these solutions were incubated at room temperature for 1 h. Wells were washed three times in 1 × PBS and 0.1% Tween 20 and bound antibodies were detected using a donkey anti-rabbit Ig HRP linked (Amersham) at a dilution of 1/1,000. Development of colorimetric reaction and measurement of optical density were performed as described above.

IgE Inhibition Assay

The ability of the Par j 1 disulphide variants to interact with IgE antibodies was determined by an ELISA inhibition experiment. A pool of sera (1/5 dilution) from 10 monosensitive Pj-allergic patients was pre-incubated overnight with increasing concentrations of each disulphide bond variant (0.25–20 µg/ml of protein). The solutions were added to the ELISA wells coated with 5 ng/ml of rPar j 1 and the ELISA steps were performed as described above. The percentage inhibition was calculated according to the following formula: inhibition (%) = $100 - OD_A/OD_B \times 100$, where OD_A and OD_B represent the optical density read with the inhibited and non-inhibited pool of sera, respectively.

Patients and Skin Prick Test

Ten patients with a clear history of Pj allergy and with skin prick test (SPT) monosensitivity to Pj commercial extract were investigated in this study. None of the patients received immunotherapy against Pj pollen and none were receiving glucocorticosteroid treatment. Allergens were used at a concentration of 1 µg/ml diluted in 0.9% NaCl. About 20 µl of the test solution was placed on the patient's forearm, with a distance of more than 2.5 cm between each prick. All tests were performed in duplicate. Histamine was used as a positive control and 0.9% NaCl solution as a negative control. Reactions were measured after 20 min. By comparison with the wheal area generated by histamine (100%), positive SPTs were divided into three classes: 4+ was assigned to SPTs with an area ≥100% of the area induced by histamine; 3+ was assigned to an area ≥80–100%, and 2+ to an area ≥50–80%. Two non-allergic subjects (P.C. and D.G.) were tested as negative controls. Each subject was informed by the investigators and signed an informed consent before the test.

Peripheral Blood Mononuclear Cell Proliferation Assay

For the proliferation assay, peripheral blood mononuclear cells (PBMCs) from 9 patients allergic to Pj pollen were cultured in triplicate at 2×10^5 cells/well in 96-well flat-bottom culture plates (Costar, Cambridge, Mass., USA) in a volume of 200 µl each. Par j 1 or the four mutated molecules were added to the culture medium as antigens at a concentration of 2 µg/ml. Cultures were pulsed after 7 days of incubation with 1 µCi of [³H]thymidine/well (Amersham Pharmacia Biotech, Uppsala, Sweden), and cells were harvested 18 h later on microbeta filter mats using a 96-well cell harvester. [³H]Thymidine incorporation was measured using a Microbeta Scintillation Counter (EG&G Wallac, Turku, Finland). The stimulation index was calculated as the quotient of the cpm obtained by allergen stimulation and the cpm obtained in unstimulated cultures (medium alone). The statistical significance of differences was evaluated using the Wilcoxon signed-ranks test.

Results

Expression and Immunological Characterisation of Disulphide Bond Variants of the Par j 1 Allergen

Data previously published by our group demonstrated the importance of the four disulphide bridges in the three-dimensional structure of the Par j 1 allergen and the importance of the Cys14–Cys29 bond in the formation of an immunodominant IgE epitope [22]. To understand in more detail the role of the cysteine residues in the interaction with the IgE, serine substitution on residues 4, 29, 30, 50 and 52 was performed. Using this strategy, we were able to generate four independent mutants: the PjA mutant lacks the Cys14–Cys29 and Cys30–Cys75 bridges; the PjB mutant lacks the Cys50–Cys91 and Cys4–Cys52 bridges; the PjC mutant lacks the Cys14–Cys29, Cys30–Cys75 and Cys4–Cys52 bridges, and the PjD mutant lacks all four bridges (fig. 1). The wild-type and mutated allergens were expressed in a prokaryotic vector containing a His tag for purification and their immunological activity was evaluated by Western blot on a 16% SDS-PAGE gel using a pool of sera (n = 30) from Pj-allergic patients who had not received any SIT. This analysis showed that the PjB mutant was still capable of binding human IgE while the PjA mutant retains only weak IgE-binding activity. The PjC and PjD mutants did not show any IgE-binding activity, suggesting that IgE recognition was dependent on the three-dimensional folding of the protein (fig. 2B). The same pool of allergic sera was used in an ELISA experiment, showing the same pattern of reaction observed in figure 2B with the PjB variant reacting as the wild-type allergen. A non-allergic serum is shown as a negative control (fig. 2A).

Reactivity of Purified Par j 1 Disulphide Bond Variants to IgE from Pj-Allergic Patients

The IgE-binding activity of the four Par j 1 disulphide bond variants was tested by ELISA using sera from 10 monosensitive Pj-allergic patients. Analysis of single sera showed a remarkable homogeneity of the reaction. In particular, the Cys4–Cys52 and Cys50–Cys91 bridges did

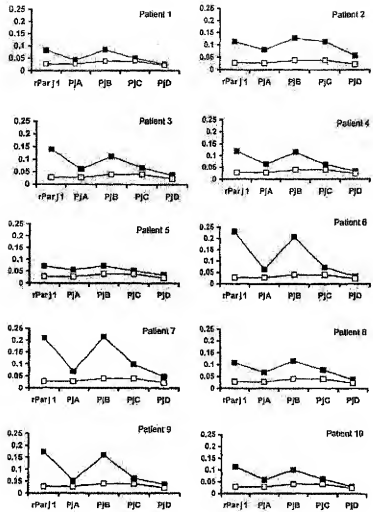

Fig. 3. ELISA detection of the IgE-binding activity of the rPar j 1 allergen and its disulphide bond variants using 10 monosensitive sera from Pj-allergic patients. Black squares indicate allergic sera; white squares indicate a non-allergic serum. The y-axis indicates optical density.

not influence the allergenicity of the protein, since this mutant (PjB) showed an IgE-binding activity comparable to the wild-type allergen. On the other hand, the Cys14–Cys29 and Cys30–Cys75 bridges seem to be crucial for IgE recognition. All the variants lacking those two bonds (PjA, PjC and PjD) presented low or even absent IgE-binding activity (fig. 3).

IgE Inhibition Assay

In order to investigate whether the disulphide bond variants were able to inhibit the binding of the IgE to rPar j 1, increasing amounts of each recombinant mutant were incubated with a pool of sera (n = 10) from monosensitive Pj-allergic patients. The data reported in table 1 suggest that all the variants lacking, at least, the Cys14–Cys29 and Cys30–Cys75 disulphide bonds exhibit a comparable low level of inhibition (about 15%). In contrast, the PjB variant (Cys50→Ser and Cys52→Ser) showed a high percentage of inhibition, retaining a substantial ability to bind human IgE (about 85%).

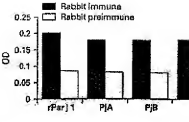

Fig. 4. ELISA detection of the Ig-binding activity of rabbit polyclonal immune and pre-immune antisera against rPar j 1. The antigens used were the wild-type rPar j 1 allergen and its disulphide bond variants.

Table 1. Inhibition of IgE binding to ELISA plate-bound rPar j 1

Allergen	Inhibition, %
rPar j 1	95
PjA	16
PjB	85
PjC	14
PjD	15

Table 2. SPT results for rPar j 1 and its disulphide bond variants

Patient	rPar j 1	PjA	PjB	PjC	PjD
1	++++	–	–	–	–
2	+++	++	+++	–	–
3	++++	–	+++	–	–
4	++++	–	++++	–	–
5	++++	++	+++	–	–
6	++++	–	+++	–	–
7	++++	–	++++	–	–
8	++++	++	+++	–	–
9	+++	–	++	–	–
10	+++	–	++	–	–
P.C.	–	–	–	–	–
D.G.	–	–	–	–	–

Rabbit Polyclonal Binding Activity

ELISA plates coated with wild-type Par j 1 and an equal amount of each recombinant disulphide bond variant were probed with an anti-rPar j 1 specific polyclonal serum to analyse their binding activity. The data obtained suggest that the PjA, PjB and PjC variants show similar behaviour, exhibiting a slight reduction in their binding ability (about 10%) compared to the Par j 1 binding. The PjD variant showed reduced binding activity (about 20%), while the pre-immune serum did not show any reactivity towards the proteins (fig. 4).

In vivo Reactivity of the rPar j 1 Disulphide Bond Variants

SPT was performed on 10 Pj-monoallergic patients who had not received SIT. All of them showed a positive cutaneous reaction to the rPar j 1 allergen. PjB was capable of inducing type I immediate hypersensitivity in 9 out of the 10 tested patients. PjA gave a positive reaction in 3 out of the 10 patients, and the wheal areas induced by the prick were reduced in size compared to those triggered by the wild-type allergen. The PjC and PjD variants did not produce any SPT reaction. No reactions were observed when non-allergic subjects were tested (table 2).

T Cell Response of the rPar j 1 Hypoallergenic Variants

PBMCs from 9 allergic patients were able to proliferate when stimulated with Par j 1, giving stimulation indices ≥ 2 (considered as the negative cut-off) and ranging from 2 to 23.5. Background proliferation in unstimulated cultures ranged between 140 and 517 cpm. When the four variants were tested as antigens, T cell reactivity was conserved in all the molecules. In response to the four mutants, PBMCs from 8 out of the 9 subjects gave stimulation indices higher than those obtained with the wild-type allergen. Stimulation index values for the 9 individual subjects are shown in table 3. The increments induced by PjA, PjC and PjD ($p < 0.02$) are statistically significant, thus indicating that the T cell reactivity was positively affected by the loss of the three-dimensional folding of the protein (table 3).

Discussion

Atopy is strictly correlated with the development of highly polarised Th2 cells characterised by IL-4, IL-5 and IL-13 cytokine production that induce B cells to switch to IgE production and promote the maturation, recruitment and activation of eosinophils.

SIT consists of repeated subcutaneous injections of increasing doses of a specific allergen, leading to a reduction in cytokine production (tolerance) or induction of 'immune deviation' from a Th2-like to a Th1-like envi-

Table 3. PBMC proliferation assay

Patient No.	Unstimulated cultures, cpm	rPar j 1	PjA	PjB	PjC	PjD
1	143	23.5	39.4	41.3	32.6	57.5
2	140	20.9	49.9	35.9	32.9	34.8
3	154	11.0	34.9	17.9	14.1	19.9
4	270	9.6	13.5	4.7	10.2	15.9
5	517	9.2	12.3	15.5	15.3	16.1
6	141	8.6	5.7	3.8	6.1	5.1
7	158	7.4	23.2	17.8	15.4	15.3
8	171	3.9	13.3	9.7	6.5	13.7
9	271	2.0	15.3	4.6	4.0	16.6
Mean	–	10.7	21.9	16.8	15.2	21.7
SEM	–	2.4	4.8	4.5	3.6	5.2

The results shown are stimulation indices, except where indicated otherwise.

ronment. Many reports have focused their attention on the molecular events involved in this transition, including the allergen concentration [24], types of antigen-presenting cells [25], the three-dimensional structure of the allergen [5] and IgE-allergen interaction [3]. Currently available pollen allergy SIT is based on crude protein extracts purified from the allergenic sources, which is associated with the risk of anaphylaxis and sensitisation to new allergens. For these reasons, Marsh et al. [26] already introduced chemical polymerisation of the allergens to produce molecules with a reduced risk of inducing systemic reactions. Their safety has been tested in animals [27], showing the efficacy of this treatment, which has been attributed to the reduced IgE reactivity. Moreover, chemical modifications of crude extracts still represent problems in terms of the standardisation and purity of the extract.

The development of recombinant DNA technology allowed the isolation and characterisation of recombinant allergens with immunological properties similar to their native counterparts, and their use might be a landmark in the design of new molecules for improved immunotherapy. A new strategy may be represented by the use of high doses of hypoallergenic recombinant molecules with no risk of side effects. In fact, a high concentration of allergen seems to modulate the T cell cytokine pattern of reaction, favouring IFN-γ production and decreasing IL-4 induction [28]. In addition, a study has reported that changes in the conformational structure of an allergen leading to non-IgE-binding molecules will activate an uptake of the allergen by a different type of antigen-presenting cell than B cells, thus inducing a balanced Th0- or Th1-like cytokine production with a decreased IgE/IgG4 ratio [5].

Different approaches have been proposed in the attempt to produce allergenic variants with reduced allergenicity [29–33]. Par j 1.0102 and Par j 2.0101 represent the two major allergenic components of Pj pollen, binding most of the Pj-specific IgE from allergic patients. They show relevant homology at the structural level, since they belong to the same family of proteins, named nsLTPs. Three-dimensional modelling by homology allowed us to identify the overall structure of the Par j 1.0101 allergen (similar folding has been shown for the Par j 2 allergen [Kennedy, D., pers. commun.]) [22]. In addition, Pj major allergens have been shown to exhibit IgE determinants spread all over the molecule with a heterogeneous pattern of recognition by individual patients [34].

For these reasons, we decided to target disulphide bridges, and developed a family of full-length three-dimensional mutants of the rPar j 1 allergen. In vivo and in vitro analysis showed that IgE-binding recognition is dependent on the three-dimensional structure of rPar j 1. In particular, site-directed mutagenesis showed the relevance of the Cys14–Cys29 and Cys30–Cys75 bridges (PjA mutant) in the antigen-IgE interaction. SPT, Western blot and ELISA showed a remarkable reduction in its allergenic activity. PjA showed very low IgE-binding activity, and only 3 out of 10 patients had cutaneous type I hypersensitivity and a reduced wheal area compared to that induced by wild-type allergen. In contrast, loss of the Cys50–Cys91 and Cys4–Cys52 bridges seems to have a minimal effect, since strong IgE-binding activity and a positive SPT were still present. This behaviour was observed when single sera or a pooled serum (n = 30) were used. Similar results were shown by ELISA inhibition, in which PjC was the only variant able to bind a large amount of Par j 1-specific IgE antibodies in solution, while the other variants exhibited very low inhibition capacity (table 1). The loss of additional disulphide bridges (PjC and PjD) leads to the absence of any IgE recognition or cutaneous reaction (fig. 2, table 2).

As an initial conclusion, it can be stated that changes in the conformation of the rPar j 1 allergen influenced the IgE epitope structure of the protein, confirming, by different techniques, the importance of discontinuous epitopes in the allergenicity of rPar j 1. Nevertheless, the reduction in the allergenicity did not influence their overall antigenicity. Using a rabbit polyclonal serum against rPar j 1, we compared the antibody binding activity of the wild-type allergen and its four variants. The hypoallergenic

variants generated by genetic engineering presented similar behaviour with a low level of reduction in their binding activity towards the anti-rPar j 1 rabbit antibodies. These data suggest that these variants still contain several protein domains similar to the native molecule and, theoretically, that they should be able to induce the production of IgG antibodies. An increasing body of evidence has shown that the induction of antibodies of IgG isotype may be crucial for successful SIT. Such blocking antibodies, which bind allergens, would prevent IgE production or suppress IgE-mediated presentation of the allergen, thus reducing T cell proliferation and the release of cytokines [see ref. 35 for a review].

In fact, T lymphocytes are the key element for the induction and maintenance of IgE production. The use of allergen-specific T cell epitopes in therapy has been tested in mice [36] and humans [37], which showed that the administration of dominant T cell peptides leads to tolerance. Even so, in atopic patients, T cell cloning studies displayed individual reactivity to T cell epitopes [38], and then the use of a single linear peptide may not be representative of the population. The use of full-length molecules should avoid this limitation. For all these reasons, we tested the Par j 1 allergen and its conformational variants by PBMC proliferation assay, and demonstrated a retained T cell reactivity with higher stimulation indices compared to the wild-type allergen, probably due to more efficient antigen presentation after the loss of the disulphide bridges [39]. In particular, the disruption of the Cys14–Cys29 and Cys30–Cys75 bridges in the PjA mutant exerts effects comparable to the mutant lacking all four bridges (PjD), suggesting that the region associated with those SH-bridges contains crucial epitopes either for antibody induction or T cell recognition. However, the mutated allergens maintain most of the T cell epitopes required for the modulation of cytokine production.

The strategy described herein is independent of the epitope sequence in itself, since it is based on the modification of the three-dimensional structure of the IgE determinants. For this reason, it is feasible for all the proteins with allergenic activity belonging to the nsLTP family (e.g. Par j 2). In fact, nsLTPs are a family of highly structurally correlated proteins which can be isolated from a wide range of plants [21]. Some of them have recently been identified as allergens in pollen and plant-derived food [40–43], so the disruption of the tertiary structure targeting the disulphide bonds should be considered as a strategy widely useful for all the allergenic members of the nsLTP family.

In conclusion, we report the immunological characterisation of a family of hypoallergenic molecules showing reduced allergenicity and retained T cell recognition. These molecules could represent a new tool in the development of a safer and more efficient therapy for Pj allergy.

References

1 Corry DB, Kheradmand F. Induction and regulation of the IgE response. Nature 1999;402 (6760 suppl):B18–B23.
2 Pirron U, Schlunck T, Prinz JC, Rieber EP. IgE-dependent antigen focusing by human B lymphocytes is mediated by the low-affinity receptor for IgE. Eur J Immunol 1990;20: 1547–1551.
3 van der Heijden FL, Joosti van Neerven RJ, van Katwijk M, Bos JD, Kapsenberg ML. Serum-IgE-facilitated allergen presentation in atopic disease. J Immunol 1993;150:3643–3650.
4 Maurer D, Ebner C, Reininger B, Fiebiger E, Kraft D, Kinet JP, Stingl G. The high affinity IgE receptor (Fc epsilon RI) mediates IgE-dependent allergen presentation. J Immunol 1995;154:6282–6290.
5 Akdis CA, Blesken T, Wymann D, Akdis M, Blaser K. Differential regulation of human T cell cytokine patterns and IgE and IgG4 responses by conformational antigen variants. Eur J Immunol 1998;28:914–925.
6 Bousquet J, Lockey R, Malling HJ. Allergen immunotherapy: Therapeutic vaccines for allergic diseases. A WHO position paper. J Allergy Clin Immunol 1998;102:558–562.
7 Durham SR, Walker SM, Varga EM, Jacobson MR, O'Brien F, Noble W, Till SJ, Hamid QA, Nouri-Aria KT. Long-term clinical efficacy of grass-pollen immunotherapy. N Engl J Med 1999;341:468–475.
8 Jutel M, Pichler WJ, Skrbic D, Urwyler A, Dahinden C, Muller UR. Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN-gamma secretion in specific allergen-stimulated T cell cultures. J Immunol 1995;154:4187–4194.
9 Saarinen H, Chelen CJ, Wen Y, Marshall JD, Umetsu DT. Allergen immunotherapy decreases interleukin 4 production in CD4+ T cells from allergic individuals. J Exp Med 1993; 178:2123–2130.
10 Chapman MD, Smith AM, Vailes LD, Arruda LK, Dhanaraj V, Pomes A. Recombinant allergens for diagnosis and therapy of allergic disease. J Allergy Clin Immunol 2000;1: 9–478.
11 D'Amato G, Spieksma FT, Liccardi G, Jager S, Russo M, Kontou-Fili K, Nikkels H, Wuthrich B, Bonini S. Pollen-related allergy in Europe. Allergy 1998;53:567–578.
12 Bass D, Baldo BA. *Parietaria* as a cause of asthma (letter). Med J Aust 1984;140:511.
13 Geraci D, Orsini U, Ruffilli A. Purification and characterization of allergens from *Parietaria officinalis* pollen. Immunochemistry 1978; 15:491–498.
14 Curti AL, Carreira J. Identification and characterization of *Parietaria judaica* allergens. Int Arch Allergy Appl Immunol 1984;74:318–323.
15 Giuliani A, Pini C, Bonini S, Mucci N, Cerroni L, Vicari G. Isolation and purification of a major allergen from *Parietaria officinalis* pollen. Allergy 1987;42:434–440.

16 Cocchiara R, Locorotondo G, Parlato A, Guarnotta G, Ronchi S, Albeggiani G, Amoroso S, Falagiani P, Geraci D: Purification of Par j I, a major allergen from *Parietaria judaica* pollen. Int Arch Allergy Appl Immunol 1989;90:84–90.

17 Costa MA, Colombo P, Izzo V, Kennedy H, Venturella S, Cocchiara R, Mistrello G, Falagiani P, Geraci D: cDNA cloning, expression and primary structure of Par jI, a major allergen of *Parietaria judaica* pollen. FEBS Lett 1994;341:182–186.

18 Duro G, Colombo P, Costa MA, Izzo V, Porcasi R, Di Fiore R, Locorotondo G, Mirisola MG, Cocchiara R, Geraci D: cDNA cloning, sequence analysis and allergological characterization of Par j 2.0101, a new major allergen of the *Parietaria judaica* pollen. FEBS Lett 1996;399:295–298.

19 Colombo P, Duro G, Costa MA, Izzo V, Mirisola M, Locorotondo G, Cocchiara R, Geraci D: An update on allergens. *Parietaria* pollen allergens. Allergy 1998;53:917–921.

20 Duro G, Colombo P, Assunta Costa M, Izzo V, Porcasi R, Di Fiore R, Locorotondo G, Cocchiara R, Geraci D: Isolation and characterization of two cDNA clones coding for isoforms of the *Parietaria judaica* major allergen Par j 1.0101. Int Arch Allergy Immunol 1997;112:348–355.

21 Poznanski J, Sodano P, Suh SW, Lee JY, Ptak M, Vovelle F: Solution structure of a lipid transfer protein extracted from rice seeds. Comparison with homologous proteins. Eur J Biochem 1999;259:692–708.

22 Colombo P, Kennedy D, Ramsdale T, Costa MA, Duro G, Izzo V, Salvadori S, Guerrini R, Cocchiara R, Mirisola MG, Wood S, Geraci D: Identification of an immunodominant IgE epitope of the *Parietaria judaica* major allergen. J Immunol 1998;160:2780–2785.

23 Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977;74:5463–5467.

24 Secrist H, DeKruyff RH, Umetsu DT: Interleukin 4 production by CD4+ T cells from allergic individuals is modulated by antigen concentration and antigen-presenting cell type. J Exp Med 1995;181:1081–1089.

25 Gajewski TF, Pinnas M, Wong T, Fitch FW: Murine Th1 and Th2 clones proliferate optimally in response to distinct antigen-presenting cell populations. J Immunol 1991;146:1750–1758.

26 Marsh DG, Lichtenstein LM, Campbell DH: Studies on 'allergoids' prepared from naturally occurring allergens. I. Assay of allergenicity and antigenicity of formalinized rye group I component. Immunology 1970;18:705–722.

27 HayGlass KT, Stefura BP: Anti-interferon gamma treatment blocks the ability of glutaraldehyde-polymerized allergens to inhibit specific IgE responses. J Exp Med 1991;173:279–285.

28 Carballido JM, Faith A, Carballido-Perrig N, Blaser K: The intensity of T cell receptor engagement determines the cytokine pattern of human allergen-specific T helper cells. Eur J Immunol 1997;27:515–521.

29 Schramm G, Kahlert H, Suck R, Weber B, Stuwe HT, Muller WD, Bufe A, Becker WM, Schlaak MW, Jager L, Cromwell O, Fiebig H: 'Allergen engineering': Variants of the timothy grass pollen allergen Phl p 5b with reduced IgE-binding capacity but conserved T cell reactivity. J Immunol 1999;162:2406–2414.

30 Ball T, Fuchs T, Sperr WR, Valent P, Vangelista L, Kraft D, Valenta R: B cell epitopes of the major timothy grass pollen allergen, Phl p 1, revealed by gene fragmentation as candidates for immunotherapy. FASEB J 1999;13:1277–1290.

31 Smith AM, Chapman MD: Reduction in IgE binding to allergen variants generated by site-directed mutagenesis: Contribution of disulfide bonds to the antigenic structure of the major house dust mite allergen Der p 2. Mol Immunol 1996;33:399–405.

32 Takai T, Yokota T, Yasue M, Nishiyama C, Yuuki T, Mori A, Okudaira H, Okumura Y: Engineering of the major house dust mite allergen Der f 2 for allergen-specific immunotherapy. Nat Biotechnol 1997;15:754–758.

33 Hakkaart GA, Aalberse RC, van Ree R: Epitope mapping of the house-dust-mite allergen Der p 2 by means of site-directed mutagenesis. Allergy 1998;53:165–172.

34 Costa MA, Duro G, Izzo V, Colombo P, Mirisola MG, Locorotondo G, Cocchiara R, Geraci D: The IgE-binding epitopes of rPar j 2, a major allergen of *Parietaria judaica* pollen, are heterogeneously recognized among allergic subjects. Allergy 2000;55:246–250.

35 Akdis CA, Blaser K: Mechanisms of allergen-specific immunotherapy. Allergy 2000;55:522–530.

36 Briner TJ, Kuo MC, Keating KM, Rogers BL, Greenstein JL: Peripheral T-cell tolerance induced in naive and primed mice by subcutaneous injection of peptides from the major cat allergen Fel d I. Proc Natl Acad Sci USA 1993;90:7608–7612.

37 Muller U, Akdis CA, Fricker M, Akdis M, Blesken T, Bettens F, Blaser K: Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom. J Allergy Clin Immunol 1998;101:747–754.

38 Dormann D, Montermann E, Klimek L, Weber B, Ebner C, Valenta R, Kraft D, Reske-Kunz AB: Heterogeneity in the polyclonal T cell response to birch pollen allergens. Int Arch Allergy Immunol 1997;114:272–277.

39 Collins DS, Unanue ER, Harding CV: Reduction of disulfide bonds within lysosomes is a key step in antigen processing. J Immunol 1991;147:4054–4059.

40 Asero R, Mistrello G, Roncarolo D, de Vries SC, Gautier M, Ciurana CL, Verbeek E, Mohammadi T, Knul-Brettlova V, Akkerdaas JH, Bulder I, Aalberse RC, van Ree R: Lipid transfer protein: A pan-allergen in plant-derived foods that is highly resistant to pepsin digestion. Int Arch Allergy Immunol 2000;122:20–32.

41 Pastorello EA, D'Ambrosio FP, Pravettoni V, Farioli L, Giuffrida G, Monza M, Ansaloni R, Fortunato D, Scibola E, Rivolta F, Incorvaia C, Bengtsson A, Conti A, Ortolani C: Evidence for a lipid transfer protein as the major allergen of apricot. J Allergy Clin Immunol 2000;105:371–377.

42 Pastorello EA, Ortolani C, Baroglio C, Pravettoni V, Ispano M, Giuffrida MG, Fortunato D, Farioli L, Monza M, Napolitano L, Sacco M, Scibola E, Conti A: Complete amino acid sequence determination of the major allergen of peach *(Prunus persica)* Pru p 1. Biol Chem 1999;380:1315–1320.

43 Sanchez-Monge R, Lombardero M, Garcia-Selles FJ, Barber D, Salcedo G: Lipid-transfer proteins are relevant allergens in fruit allergy. J Allergy Clin Immunol 1999;103:514–519.

Exhibit 2

cDNA cloning, sequence analysis and allergological characterization of Par j 2.0101, a new major allergen of the *Parietaria judaica* pollen

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Abstract A clone (P2) coding for an allergen of *Parietaria judaica* (Pj) pollen has been isolated and sequenced from a cDNA library in lambda ZAP using a pool of 23 sera from Pj-allergic patients. The clone contained an insert of 622 nucleotides with an open reading frame of 133 amino acids (aa) and a putative signal peptide of 31 aa giving a deduced mature processed protein of 102 aa with a molecular mass of 11 344 Da. The expressed recombinant protein, named rPar j 2.0101, was a major allergen since it reacted with IgE of 82% (23/28) of the sera of Pj-allergic subjects analyzed. It was shown to be a new allergen since (i) the amino acid sequence homology with the already reported recombinant allergen Par j 1.0101 was 45% and (ii) there was no cross-inhibition between rPar j 2.0101 and rPar j 1.0101. In addition, rPar j 2.0101 inhibited 35% of the specific IgE for 10–14 kDa native allergens and preincubation of sera from Pj-allergic patients with both rPar j 2.0101 and rPar j 1.0101 fully abolished the IgE recognition of the 10–14 kDa native allergen region, suggesting that these two allergens contributed to the region.

Key words: cDNA cloning; Allergy; Immunoglobulin E; *Parietaria judaica*

1. Introduction

Parietaria judaica pollen is the main cause of allergy in the Mediterranean area and an epidemiological study carried out on allergic patients in Sicily showed that up to 50% reacted to Pj pollen extract [1,2]. The allergenic molecules contained in the pollen are usually a complex mixture of various proteins differing in either specific IgE binding or biochemical properties. The Pj pollen extract contains at least nine allergens having different molecular weights and IgE-binding specificity. The molecular mass ranges between 10 and 80 kDa [1–5] and the allergens found on the 10–14 kDa region reacted with the IgE of 95% of the sera from Pj-allergic patients tested, suggesting that major allergens are present in this region [6]. In order to plan a diagnostic and therapeutic approach to the allergic reaction, a preliminary step is to purify and to characterize each major allergen and a powerful tool towards this target is the molecular cloning of the allergens [7,8]. We have already cloned and characterized one of the major allergens of the *P. judaica* pollen that belongs to the 10–14 kDa region [9], Par j 1.0101, a protein of 139 amino acids and a predicted molecular mass of 14 509 Da. In this paper, we report the cloning, sequence, expression and allergenic activity of a new major allergen of Pj pollen named Par j 2.0101 according to the suggested allergen nomenclature [10].

2. Materials and methods

2.1. Total and poly(A)$^+$ RNA preparation

Total and poly(A)$^+$ RNA extracted from Pj flowers collected in Palermo, Italy, was prepared as described [9].

2.2. cDNA library screening and cDNA sequencing

Double-stranded cDNA was synthesized from mRNA by using oligo(dT) primers and cloned into the *Eco*RI and *Xho*I sites of the lambda ZAP expression vector, according to the Stratagene cDNA cloning kit (Stratagene, USA).

6×10^5 plaques were immunologically screened by using a pool of sera ($n=23$) diluted 1:5 in PBS-containing 0.25% BSA, obtained from patients allergic to Pj pollen. Positive clones were identified by using ^{125}I-labelled anti-human IgE.

The nucleotide sequence of the cDNA insert was determined on both strands of the PBK-CMV plasmid vector by the dideoxy chain termination method using a Sequenase Kit (Amersham, USA).

2.3. Cloning in pMALc2

The oligonucleotides were synthesized by Pharmacia Biotech. Primers for PCR were (lower-case letters indicate the restriction enzyme cloning site; upper-case letters denote the coding sequence): (oligo 1) 5′ ccggaattcGAGGCTTGCGGGAAAGTGGTGCAGGAT 3′ (oligo 2) 5′ gtgctagaATAGTAACCTCTGAAAATAGTACTTTGG 3′. 1 ng of the P2 clone was subjected to 30 cycles of PCR under the following conditions: 94°C 30 s, 52°C 30 s, 72°C 30 s.

The PCR product was fractionated on 1.8% agarose gel and after several steps of purification, digested with *Eco*RI and *Xho*I restriction enzymes. The fragments were cloned in the *Eco*RI/*Xba*I sites of the pMALc2 vector (Biolabs, UK). Recombinant clones were sequenced using the dideoxynucleotide chain termination method.

2.4. Preparation of the recombinant allergens

The recombinant clones were grown to 0.5–0.6 OD_{600} in LB broth and induced for 2 h with 0.3 mM isopropylthio-β-D-galactoside (IPTG). The cells were harvested by centrifugation (4000 rpm/20 min) and stored frozen. After thawing, the pellet was dissolved in sodium phosphate buffer (10 mM Na phosphate, pH 7.2; 200 mM NaCl, 1 mM EDTA, 1 mM NaN_3) and lysed by sonication using a Heat-System-Ultrasonic, Inc/W-385. The cell debris was then removed by centrifugation (9000 rpm/30 min) and the supernatant was diluted 1:20 with 10 mM EDTA and concentrated up to 2 mg/ml of total proteins by using a centriprep concentrator (Amicon) with a molecular mass cut-off of 10 kDa. The concentration of the recombinant allergens was detected by densitometry analysis of SDS-PAGE gels stained with Coomassie Brilliant Blue.

2.5. Western blot analysis

Protein samples were denatured under reducing conditions by boiling for 5 min in 50 mM Tris-HCl pH 6.8, 1% SDS, 2% β-mercap-

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Abbreviations: Pj, *Parietaria judaica*; IgE, immunoglobulin E; RAST, radioallergosorbent test; cDNA, DNA complementary to mRNA; PCR, polymerase chain reaction.

EMBL data bank accession number P2:X95865.

PII S0014-5793(96)01328-2

```
                                                    M  R  T  V  S  M  A  A
P.j 2.0101  acagtctcagacacacccctttccaaacttccaacATGAGGACCGTGTCGATGGCGGCA
            1                                                          60

            L  V  V  I  A  A  A  L  A  W  T  S  S  A  E  P  A  P  A  P
            CTCGTTGTGATCGCGGCGGCGCTCGCGTGGACATCTTCGGCTGAGCCGGCTCCAGCCCCG
                                                                      120

            A  P  G  E  E  A  C  G  K  V  V  Q  D  I  M  P  C  L  H  F
            GCCCCAGGAGAGGAGGCTTGCGGGAAAGTGGTGCAGGATATAATGCCGTGCCTGCATTTC
                                                                      180

            V  K  G  E  E  K  E  P  S  K  E  C  C  S  G  T  K  K  L  S
            GTGAAGGGGAGGAGAAGGAGCCGTCGAAGGAGTGCTGCAGCGGCAGGAAGAAGCTGAGC
                                                                      240

            E  E  V  K  T  T  E  Q  K  R  E  A  C  K  C  I  V  R  A  T
            GAGGAGGTGAAGACGACGGAGCAGAAGAGGGAGGCCTGCAAGTGCATAGTGCGCGCCACG
                                                                      300

            K  G  I  S  G  I  K  N  E  L  V  A  E  V  P  K  K  C  D  I
            AAGGGCATCTCCGGTATCAAAAATGAACTTGTCGCCGAGGTCCCCAAGAAGTGCGATATT
                                                                      360

            K  T  T  L  P  F  I  T  A  D  F  D  C  S  K  I  Q  S  T  I
            AAGACCACTCTCCCGCCCATCACCGCCGACTTCGACTGCTCCAAGATCCAAAGTACTATT
                                                                      420

            F  R  G  Y  Y  *
            TTCAGAGGTTACTATtagcaagttagagagcgttttctcttaatttctaagtgtggggaa
                                                                      480

            ctaaaaatgaaattatggcatgttacgttcctataaggccatgttctttatatgaataatga
                                                                      540

            tgtattatgtgtgtaattgatattgatgtaatgttaacaatacgatggaataatgtt
                                                                      600

            aacaatatgaaattgctactctaaaaaaaaaaaaaaaa 3'
```

Fig. 1. Nucleotide and deduced amino acid sequence of the cDNA coding for the rPar j 2.0101 allergenic protein. Numbers in the right margin refer to the position of the nucleotides. The deduced amino acid sequence is shown using the single-letter code above the corresponding nucleotide triplet. The primers used for PCR are underlined.

(A)

```
                    1                                                  25
Par j 2.0101        M R T V S M A A L V V I A A A L A W T S S A E P A
                    26                                                 50
Par j 2.0101        P A P A P G E E A C G K V V Q D I M P C L H F V K
Par j 1.0101                  Q - T - - T M - R A L - - - - F - - Q
                    51                                                 75
Par j 2.0101        G E E K E P S K E C C S G T K K L S E E V K T T E
Par j 1.0101        - K - - - - - - G - - - - A - R - D W - T - - G P
                    76                                                100
Par j 2.0101        Q K R E A C K C I V R A T K G I S G I K N E L V A
Par j 1.0101        - R V H - - E - - Q T - M - T Y - D - D G K - - S
                    101                                               125
Par j 2.0101        E V P K K C D I K T T L P P I T A D F D C S K I Q
Par j 1.0101        - - - - E - G - V D S K L P P I D V N M D C - T L
                    126                                               150
Par j 2.0101        S T I F R G Y Y *
Par j 1.0101        G V V P - Q P Q L P V S L R H G P V T G P S D P A

Par j 1.0101        H K A R L E R P Q I R V P F F A P E K A *
```

(B)

```
                    34                                  50
Par j 1B            G P K G K V V K R I M P C L K F V K G
                      | | | |   | | | | |   | | | |
Par.j 2.0101        E A C G K V V Q D I M P C L H F V K G
                    |   | | | | | | | | |   | | | |
Par j 1A            A D G K V V Q D I M P P L L F V K
```

Fig. 2. (A) Comparison between the rPar j 2.0101 and rPar j 1.0101 allergens, the putative signal peptide is underlined. (B) Comparison between the rPar j 2.0101 and two native isoforms of *Parietaria* allergens, i.e. Par 1A and Par 1B [5]. The dash indicates identity. Numbers refer to the amino acid sequence position of the Par j 2.0101 allergen.

methanol, 0.01% bromophenol blue and separated by SDS-PAGE. The proteins were therefore electroblotted to Immobilon-P membranes (Millipore, USA) and then blocked for 3 h at room temperature in PBS supplemented with 3% BSA, 0.5% Tween and 0.02% NaN_3 and washed three times with PBS containing 0.1% (v/v) of Tween 20. Inhibition was carried out by adding to the diluted serum pool increasing amounts of recombinant proteins for 3 h at room temperature. The filters were then incubated overnight at room temperature with the serum pool previously absorbed. After a washing step, the filters were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated rabbit anti-human IgE (Sigma, St. Louis, MO). The final reaction was developed with an ECL detection system (Amersham, USA). The signal intensity was measured by using a Biorad densitometer (model GS-670).

3. Results and discussion

Ten clones capable of interacting with human IgE were isolated after screening of a lambda ZAP cDNA library using a pool of 23 sera of patients allergic to *P. judaica* pollen. Seven clones were still capable of binding human IgE after several steps of purification. The purified clones gave no IgE binding when tested with sera ($n = 5$) of subjects not allergic to Pj pollen. The sequence of the clones was then analyzed and one clone, named P2, was found to share an independent full-length cDNA. Sequence analysis of the P2 clone showed a 622 nucleotide insert with an open reading frame of 399 bp terminating with a TAG stop codon (Fig. 1). The cDNA contained a 36 nucleotide leader sequence and 184 nucleotide untranslated region with a canonical polyadenylation site and poly(A) tail. The cDNA insert encoded a protein of 133 aa with a deduced molecular mass of 14 105 Da. The hydrophobicity profile of the protein showed, in the amino-terminal region, a putative signal peptide of 31 aa specific for eukaryotic glycosylated proteins, giving a deduced mature processed protein of 102 aa with a molecular mass of 11 344 Da.

The recombinant allergenic protein named rPar j 2.0101 showed an homology of 45% with the aa sequence of the

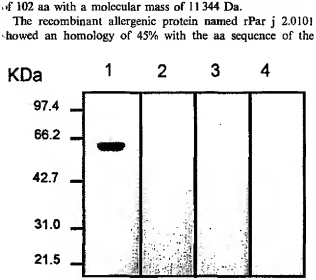

Fig. 3. Western blotting analysis. The recombinant allergen Par j 2.0101 (1 μg/lane) was run on 10% SDS-PAGE and immunoblotted as described in Section 2. Molecular mass markers (Biorad, USA) expressed in kDa are shown on the left. Lanes: 1, induced rPar j 2.0101 incubated with a pool of sera from Pj-allergic patients; 2, induced rPar j 2.0101 incubated with a pool of sera from non-allergic subjects; 3, non-induced rPar j 2.0101 incubated with a pool of sera from Pj-allergic patients; 4, induced pMAL-c2 incubated with a pool of sera from Pj-allergic patients. The pool of sera was diluted 1:5 with PBS-0.25% BSA. Specific IgE binding was detected with the HRP-conjugated anti-human IgE and ECL system.

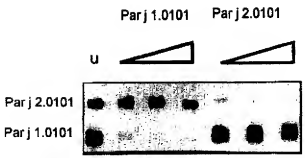

Fig. 4. Slot-blot inhibition. The recombinant allergens Par j 2.0101 and Par j 1.0101 were spotted (1 μg/slot) on nitrocellulose. The pool of sera from Pj-allergic patients diluted 1:5 was preincubated for 3 h at room temperature, with increasing amounts of recombinant allergen (1, 10, 50 μg/ml). U: pool of sera diluted 1:5 and preincubated with buffer alone as a control. Specific IgE binding was detected as reported in Fig. 3.

recombinant allergen Par j 1.0101 already reported [9] (Fig. 2A). In addition, from amino acids E32 to G51, rPar j 2.0101 showed significant homology (Fig. 2B) with the amino-terminal region of two native isoallergens, Par j 1A and Par j 1B, isolated by monoclonal antibody-based affinity chromatography and partially sequenced [5].

In order to achieve characterization at the molecular and immunological levels, the cDNA of the P2 clone after PCR was cloned without the putative signal peptide in the pMALc2 expression vector, giving a fused protein with a molecular mass of approx. 53 kDa. rPar j 2.0101 expressed as a protein fused to maltose binding protein (MBP), was capable of binding specifically the IgE from sera of patients allergic to *P. judaica* pollen, as shown by Western blot analysis (Fig. 3). The allergologic relevance of the recombinant Par j 2.0101 was determined by slot-blot analysis [11] using single sera ($n = 28$) from patients allergic to Pj pollen with a high level (RAST class 4+) of specific IgE. 82% (23/28 sera) of the sera showed IgE capable of binding the recombinant allergen Par j 2.0101, therefore, it can be classified as a major allergen (data not shown).

In order to assess the allergenic cross-reactivity between the recombinant allergens Par j 2.0101 and Par j 1.0101, the proteins were spotted on nitrocellulose membranes and incubated with a pool of 23 sera from Pj-allergic patients. Preincubation of the serum pool with increasing amounts of rPar j 2.0101 or rPar j 1.0101 completely abolished specific IgE binding to the same allergen without interference with the other and the results demonstrated in Fig. 4 strongly suggested that the two allergens showed a different IgE epitope composition.

The recombinant allergen Par j 2.0101 was capable of inhibiting approx. 35% of specific IgE binding to the 10–14 kDa native allergens group (Fig. 5). When preincubation of the serum pool was performed with both the recombinant Par j 2.0101 and Par j 1.0101, the binding of IgE to the 10–14 kDa native allergen region was totally inhibited, strongly suggesting that only those two allergens contributed to that region and that together they were capable of inhibiting the majority of the IgE specific for Pj allergens (Fig. 5).

Several isoforms of the major allergen Par j 1.0101 have been isolated and characterized (manuscript in preparation), while work is actually in progress in order to isolate isoforms for Par j 2.0101 as well.

Finally, a search made at the EMBL data bank showed that

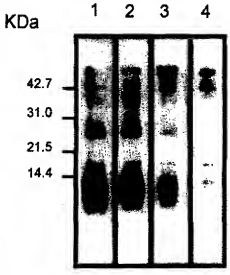

Fig. 5. Western blotting inhibition. The Pj pollen crude extract (5 μg/lane) was separated on 16% PAGE-SDS and immunoblotted as described in Section 2. Markers (Bio-Rad) expressed in kDa are shown on the left. Lanes: 1, serum pool preincubated with buffer alone as a control; 2, serum pool preincubated with 50 μg/ml of rPar j 2.0101; 3, serum pool preincubated with 50 μg/ml of rPar j 1.0101; 4, serum pool preincubated with 50 μg/ml of rPar j 2.0101+rPar j 1.0101. The inhibition procedure and IgE binding detection were performed as described in Figs. 3 and 4.

the recombinant allergen rPar j 2.0101 belongs to a family of protein referred to as non-specific lipid transfer protein (ns-LTPs) [12,13]. These proteins constitute a broad family capable of transferring lipid molecules through membranes.

In conclusion, in the present paper we have described the cloning and sequencing of a new allergen named Par j 2.0101. It is a major allergen since it was found to be capable of interacting with IgE of 82% of the Pj-allergic sera tested. Regarding the already cloned major Pj allergen rPar j 1.0101, rPar j 2.0101 is a different allergenic protein for the reason that it showed a different amino acid sequence and because there was no evident competition between the two recombinant allergens.

References

[1] Geraci, D., Billesbolle, K.B., Cocchiara, R., Lowenstein, H. and Ipsen, H. (1985) Int. Arch. Allergy Appl. Immunol. 78, 421–428.
[2] Ford, S.A., Baldo, B.A., Geraci, D. and Bass, D. (1986) Int. Arch. Allergy Appl. Immunol. 79, 120–126.
[3] Cocchiara, R., Locorotondo, G., Parlato, A., Guarnotta, G., Ronchi, S, Albeggiani, G., Amoroso, S., Falagiani, P. and Geraci, D. (1989) Int. Arch. Allergy Appl. Immunol. 90, 84–90.
[4] Polo, F., Ayuso, R. and Carreira, J. (1991) Mol. Immunol. 28, 169–175.
[5] Ayuso, R., Carreira, J., Lombardero, M., Duffort, O., Peris, A., Basombs, A. and Polo, F. (1993) Mol. Immunol. 30, 1347–1354.
[6] Kahlert, H., Weber, B., Teppke, M., Wahl, R., Cromwell, O. and Fiebig, H. (1996) Int. Arch. Allergy Appl. Immunol. 109, 141–149.
[7] Perez, M., Ishioka, G.Y., Walker, L.E. and Chesnut, R.W. (1990) J. Biol. Chem. 265, 16210–16215.
[8] Chua, K.Y., Stewart, G.A., Thomas, W.R., Simpson, R.J., Dilworth, R.S., Piozza, T.M. and Turner, K.J. (1988) J. Exp. Med. 167, 175–182.
[9] Costa, M.A., Colombo, P., Izzo, I., Kennedy, H., Venturella, S., Cocchiara, R., Mistrello, G., Falagiani, P. and Geraci, D. (1994) FEBS Lett. 341, 182–186.
[10] King, T.P., Hoffman, D., Lowenstein, H., Marsh, D.G., Platts-Mills, T.A. and Thomas, W. (1994) Int. Arch. Allergy Immunol. 105, 224–233.
[11] Barbieri, R., Duro, G., Costa, M.A. and Izzo, V. (1994) Anal. Biochem. 216, 461–462.
[12] Tchang, F., This, P., Stiefel, V., Arondel, V., Morch, M.D., Pages, M. et al. (1988) J. Biol. Chem. 263, 16849–16855.
[13] Vignols, F., Lund, G., Pammi, S., Tremousaygue, D., Grellet, F., Kader, J.C., Puigdomenech, P. and Delseny, M. (1994) Gene 142, 265–270.